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INSTANT CIRRHOSIS

AN IMPROVED METHOD FOR PRODUCING CIRRHOSIS OF THE LIVER IN RATS BY SIMULTANEOUS ADMINISTRATION OF CARBON TETRACHLORIDE AND PHENOBARBITONE

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SUMMARY.—An improved method for production of cirrhosis of the liver in rats is described. Rats are given CCl_4 and phenobarbitone simultaneously. The method doubles the yield of cirrhotic animals and halves the time taken for their production, in comparison with CCl_4 alone.

CCl_4 inhalation results in fewer intercurrent deaths than oral dosing of CCl_4. The cirrhosis as observed histologically is accompanied by gross nodularity of the liver, splenomegaly, portal hypertension and testicular atrophy. About 50 per cent of animals have measurable ascites. Animals killed during application of the new method show that cirrhosis develops only after 4 weeks of CCl_4 dosage.

IT has long been known that cirrhosis of the liver can be produced in rats by dosing them twice weekly with carbon tetrachloride, (CCl_4), (Cameron and Karunaratne, 1936; Forbes, 1939; Oliver and Sutton, 1966).

In our hands, this method has serious drawbacks. It requires a long time, many animals die intercurrently, and a proportion of the rats that survive do not develop cirrhosis even after prolonged dosage.

We have shown that the acute toxicity of CCl_4 is dependent upon its metabolism by microsomal hydroxylating enzyme systems of the liver, and toxicity is therefore enhanced by phenobarbitone and other inducers of these systems (McLean and McLean, 1966; Garner and McLean, 1969). In stock rats the LD_{50} for CCl_4 is about 5 ml./kg. body weight, while in phenobarbitone-treated rats it falls to 0·5 ml./kg.

The following experiments were set up to see if simultaneous treatment with CCl_4 and phenobarbitone would give a more rapid and reliable yield of cirrhosis of the liver.

MATERIALS AND METHODS

Rats.—Male rats weighing 100–150 g. (CFE strain, Carworth Farms, Alconbury, Herts., or Tuck Farms, Wistar strain) were maintained on a stock pellet diet (41B).

Phenobarbitone water.—Sodium phenobarbitone was dissolved in tap or distilled water at a concentration of 0·5 g./l. This was the only drinking water available to the rats (Marshall and McLean, 1969).

CCl_4 inhalation.—A wooden box, with a glass front (72 × 45 × 46 cm., *i.e.* 150 l. capacity) was fitted with an inlet tap and housed in a fume cupboard. Compressed air was passed, *via* a flow meter, at 2 l./min., bubbling through a train of two wash bottles containing

" Analar " CCl_4 maintained at 20°, and into the box. Air left the box by leaking through the joints. At each dose the CCl_4 vapour was run in for a given number of minutes and was then turned off and the rats left in the box for an equal number of minutes. For a 45 l. box 1 l./min. of saturated air was blown in.

Dosing schedule.—Rats given CCl_4 alone were placed in the inhalation chamber for 20 min. twice a week. Carbon tetrachloride was blown in for 10 min., and the rats then left in the chamber for further 10 min., by which time they were lightly anaesthetised by the CCl_4.

For the CCl_4 + phenobarbitone group, the rats were given phenobarbitone alone for one week and then exposed to CCl_4 for a total of 4 min. on the first gassing, 6 min. at the second and third gassings, and 10 min. thereafter (*i.e.* CCl_4 blown in for 5 min., the rats left in the chamber for a further 5 min. and then taken out). CCl_4 dosage was stopped after 8 weeks.

The optimum dosing schedule depends on the concentration of CCl_4 achieved and hence on the size of the box. It was found that a dose which allowed 80 per cent of the rats to survive the first 3 weeks gave the best results.

Rats were killed at intervals during, and after, various dosing schedules where CCl_4 was given orally or by inhalation, with and without phenobarbitone.

Histology.—Slices of liver about 3 mm. thick were cut from all animals and fixed in formol saline. Sections were cut and stained with haematoxylin and eosin, reticulin, Van Gieson, and periodic acid Schiff, stains.

Other measurements.—Liver and spleen weights were measured and the presence of ascites recorded. The splenic blood pressure was recorded in one group.

RESULTS

Group 1. *Rats inhaling CCl_4 + phenobarbitone in drinking water* (8 *weeks dosage*)

In a number of experiments on this schedule, survival at 8 weeks ranged from 40–85 per cent. The rest died during the course of dosage, some in the first 3 weeks with severe, acute liver injury and some at 6 weeks and later with gross ascites, hydrothorax and jaundice. The animals in the late group had grossly nodular livers on naked eye inspection.

TABLE.—*Dosage Schedules*

Group	CCl_4	Phenobarbitone
1	16 doses by inhalation	In drinking water 1 week prior to first inhalation and throughout
2	16–48 doses by inhalation	None
3	16 doses orally	In drinking water 1 week prior to first CCl_4 dose and throughout
4	12–26 doses orally	None
5	16 doses by inhalation	In drinking water 1 week prior to first inhalation and throughout

Histology.—In one experiment starting with 24 rats, 22 survived 8 weeks dosage—of these, 19 showed moderate to severe portal cirrhosis with generalised fibrosis, periportal chronic inflammation, bile duct proliferation and destruction of normal liver architecture with the formation of regeneration nodules (i.e. islands of hepatic parenchyma lacking a proper micro-anatomical relationship to either central veins or portal tracts). The other 3 were judged to show severe chronic hepatitis, *i.e.* the stage prior to the establishment of cirrhosis, in which there is considerable fibrosis and chronic inflammation, but without loss of the basic architecture to form nodules.

In experiments with a higher mortality, the histological picture showed more severe cirrhosis in all survivors (see Group 5).

Group 2. Rats given CCl_4 inhalation without phenobarbitone

After 8 weeks (16 doses), the livers remained normal, whereas by 15 weeks (30 doses), the hepatic changes were those of a chronic hepatitis in which there was inflammation and fibrosis around the portal tracts, but without true regeneration nodules. Only after 24 weeks did the livers show a true cirrhosis, in which a generalised fibrosis and nodule formation were obvious, and even at this stage the degree of cirrhosis varied considerably, from mild to severe in different animals.

Group 3. Rats given oral CCl_4 and phenobarbitone

Rats (12) were given oral CCl_4 0·2 ml./kg., orally twice a week. Of these 6 died in the first few weeks. The animals (4) that died after 5–7 weeks (10–14 doses) showed severe cirrhosis. The 2 survivors sacrificed 1 week after surviving 8 weeks of CCl_4 dosage showed chronic hepatitis which had not yet become cirrhosis.

Group 4. Rats given oral CCl_4 alone

A group of 12 rats were given 1 ml. CCl_4/kg. orally, twice a week, without phenobarbitone. Most of these rats died early in the experiment. One rat died after 6 weeks with mild cirrhosis, whereas another killed after 15 weeks had only a mild chronic hepatitis.

Group 5. Time course of the development of cirrhosis

A group of 45 rats were given phenobarbitone and CCl_4 inhalation (as in Group 1).

Two or 3 animals were killed 3 days after each gassing in the first few weeks and thereafter at weekly intervals, just before the next dose of CCl_4 was due. Even after just 2 doses of CCl_4 the portal tracts were expanding with proliferation of both fibroblasts and bile ducts, as well as inflammatory cell infiltration. After only 4 gassings (2 weeks) active granulation tissue linked up the portal tracts with the centrilobular zones, and by 5 doses of CCl_4, the histological picture was that of severe chronic hepatitis (borderline early cirrhosis) (Fig. 1). After the eighth dose (4 weeks), the animals all showed cirrhosis (Fig. 2), which then became progressively more severe with continuation of treatment (Figs. 3 and 4). Treatment with CCl_4 was stopped after 16 doses (8 weeks).

EXPLANATION OF PLATES

FIG. 1.—Rat liver. Oral phenobarbitone + CCl_4 inhalation for 2½ weeks (5 doses). Reticulin fibres link centrilobular and portal zones. Reticulin × 27.

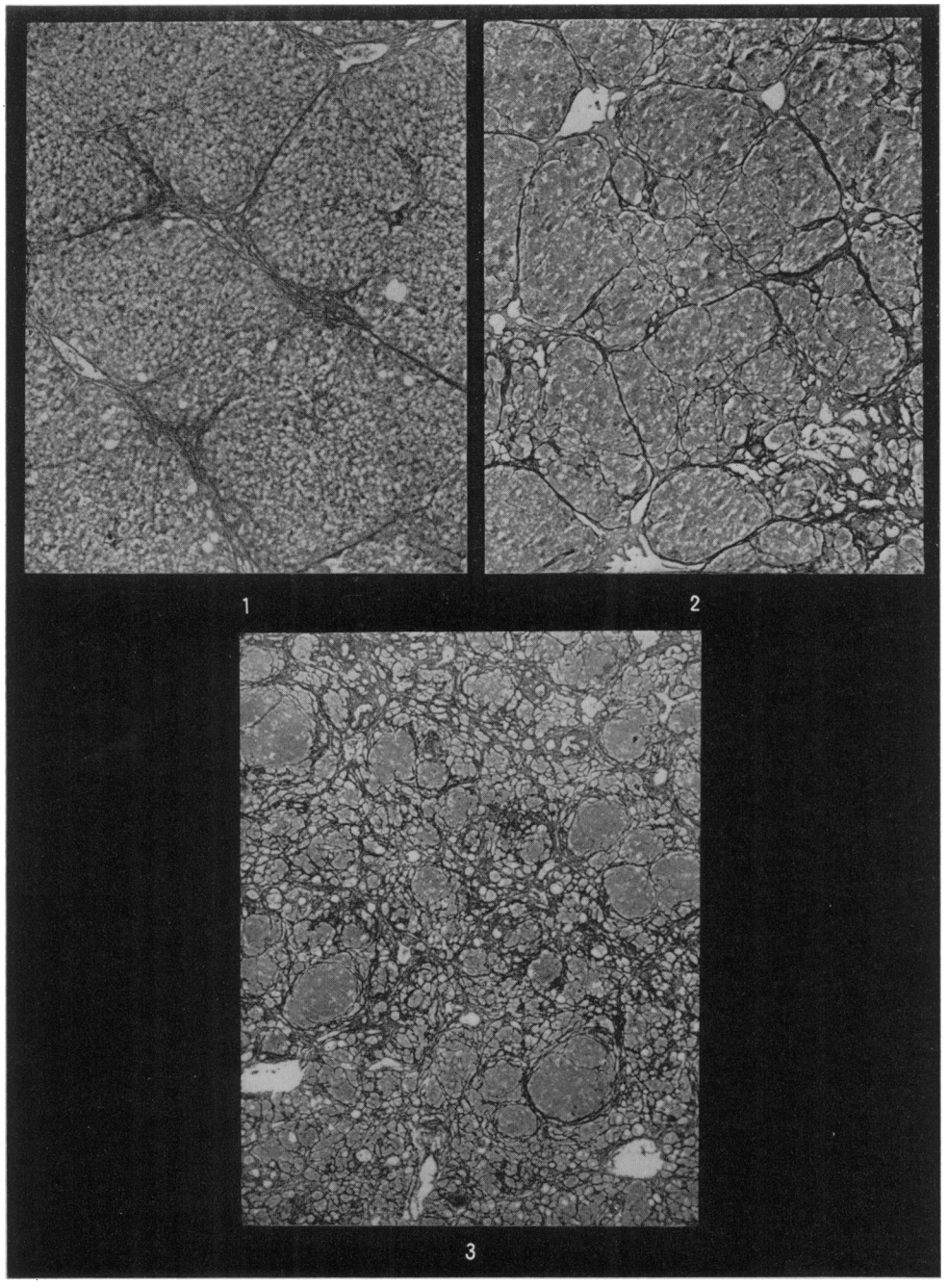
FIG. 2.—Rat liver. Oral phenobarbitone + CCl_4 inhalation for 4 weeks (8 doses). Cirrhosis. Reticulin × 27.

FIG. 3.—Rat liver. Oral phenobarbitone + CCl_4 inhalation for 8 weeks (16 doses), last dose given 8 days previously. Cirrhosis more severe than at 4 weeks. Reticulin × 27.

FIG. 4.—As in Fig. 3 but stained H. and E. × 27. Vacuolated and fatty cells are present in many small nodules.

FIG. 5.—Rat liver. Oral phenobarbitone + CCl_4 inhalation for 8 weeks (16 doses). Last dose of CCl_4 given 56 days previously. There are now more large nodules and fewer small ones. Reticulin × 27.

FIG. 6.—As in Fig. 5 but PAS stain × 68. Nodules containing glycogen are adjacent to nodules without glycogen.



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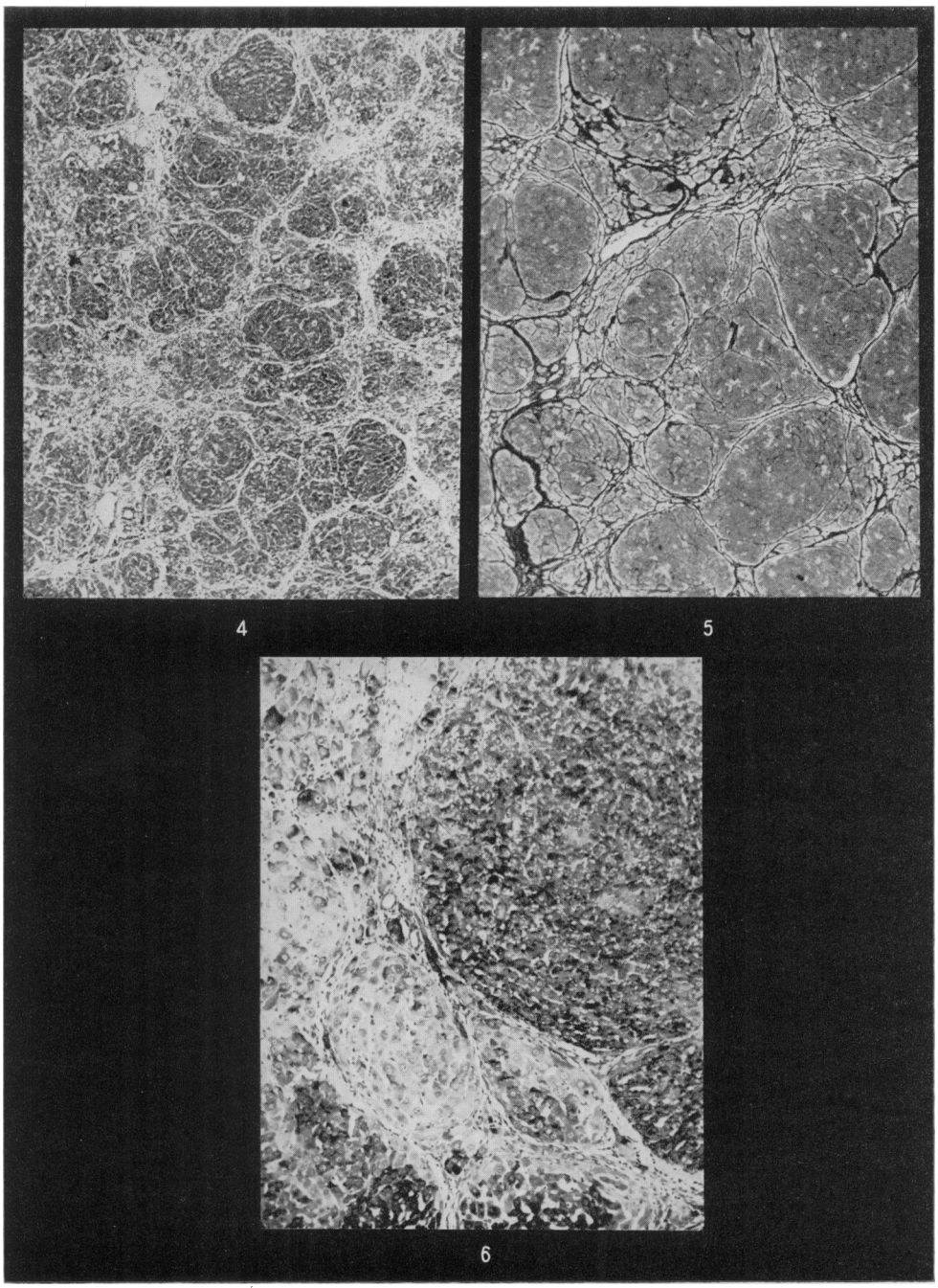

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Other observations on rats given CCl_4 inhalation + phenobarbitone

Ascites was first noted after 10 doses, and was thereafter found in over half the animals. Ascites was still present in some rats killed 8 weeks after the last (16th) dose. The amount of ascites fluid varied from 1–50 ml., and even when no free fluid was present, oedema of the omentum was frequently observed.

Ascites was usually accompanied by hydrothorax, which was sometimes so severe as to impede respiration, and was probably the cause of death in many animals.

Ascites was not noted in the rats that had received CCl_4 alone, without phenobarbitone, in the present experiments.

Splenic enlargement was first found after 4 doses of CCl_4 in the +phenobarbitone groups (Group 5) and was uniformly present after 7 doses. The spleen weight then varied from 1–2 g., while in control animals the spleen weighed less than 0·7 g.

In some very cirrhotic rats the testes were atrophic and shrunken to half the weight found in control rats.

Splenic pressure

Five rats had splenic blood pressure measured a week after the 16th dose of CCl_4. The control value and the value at intervals after a single dose of CCl_4 (McLean and Hill, 1969), was $13{\cdot}5 \pm 1{\cdot}5$ cm. saline. In the cirrhotic rats, the pressures were 27, 19, 18, 21 and 23 cm. saline. In some instances collateral veins could be seen around the oesophagus and some rats died with their intestines full of blood or with rectal bleeding.

Recovery after 16 doses of CCl_4 + phenobarbitone

In the first 10 days after cessation of CCl_4 dosage, histological signs of acute cell damage similar to the damage following a single dose of CCl_4 could still be observed. Vacuolation, hydropic cells, cells containing lipid droplets, and degenerating cells, could be found in many nodules. Cell nuclei of variable size and staining characteristics were also found.

Glycogen stains showed that only after 2 weeks of CCl_4 were any nodules found containing any glycogen, while at that time many nearby nodules or isolated cells contained no glycogen.

After 8 weeks, the glycogen distribution was sometimes still patchy, while in other livers there was a uniform, plentiful amount of glycogen in all nodules. By 8 weeks after cessation of CCl_4 dosage, the proportion of large nodules with normal looking cells, arranged in regular columns, with evenly sized nuclei, had increased and the relative area occupied by small nodules had decreased (Figs. 5 and 6).

Liver water content which was high throughout CCl_4 dosage, slowly sank on recovery (Marshall and McLean, unpublished).

DISCUSSION

These experiments show that addition of phenobarbitone to the drinking water during the induction of cirrhosis by CCl_4 greatly increases the yield of cirrhotic animals and halves the time taken to produce the cirrhosis. The rats

have splenomegaly, portal hypertension and testicular atrophy and over 50 per cent develop measurable ascites. On all these counts they seem to be in a condition comparable with human cirrhosis.

The method is easy to use, with a single proviso, that the first 6 doses must be carefully adjusted to obtain about 80 per cent of survivors at this time. Milder dosing reduces the severity of cirrhosis, while heavier dosing sharply reduces the number of eventual survivors. Later doses are not critical.

Marshall and McLean (1969), have shown that administration of phenobarbitone in the drinking water causes a steady and prolonged rise in the amount of cytochrome P 450 in rat liver. Evidence is accumulating (Garner and McLean, 1969), to suggest that CCl_4 is metabolised in the liver, into a toxic substance by a microsomal system, whose activity is tied to the P 450 level. A rise in microsomal P 450 is equivalent, in our animals, to an increase in the dose of CCl_4 " toxin ". This is the situation that applies to the first CCl_4 dose, after 1 week on phenobarbitone water. At later doses the situation is more complex. The P 450 activity in the liver is still being raised by the phenobarbitone but has also been decreased by the necrosis following the previous dose (Dingell and Heimberg, 1968). The lethal action of the inhaled CCl_4 dose therefore varies as the sum of these two influences on the P 450 varies.

Phenobarbitone not only causes an increase in microsomal enzyme activity, but also causes a massive growth of the liver. The liver weight may increase by 50 per cent and liver cell numbers increase (Kunz, Schaude, Schmid and Siess, 1966). It is possible that the simultaneous application of the damaging agent (CCl_4), and the stimulus to growth, also favours the development of cirrhosis.

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